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Spatial and temporal expression of zebrafish glutathione peroxidase 4 a and b genes during early embryo development

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ABSTRACT

Antioxidant cellular mechanisms are essential for cell redox homeostasis during animal development and in adult life. Previous *in situ* hybridization analyses of antioxidant enzymes in zebrafish have indicated that they are ubiquitously expressed. However, spatial information about the protein distribution of these enzymes is not available. Zebrafish embryos are particularly suitable for this type of analysis due to their small size, transparency and fast development. The main objective of the present work was to analyze the spatial and temporal gene expression pattern of the two reported zebrafish glutathione peroxidase 4 (GPx4) genes during the first day of zebrafish embryo development. We found that the *gpx4b* gene shows maternal and zygotic gene expression in the embryo proper compared to *gpx4a* that showed zygotic gene expression in the periderm covering the yolk cell only. Following, we performed a GPx4 protein immunolocalization analysis during the first 24-h of development. The detection of this protein suggests that the antibody recognizes GPx4b in the embryo proper during the first 24 h of development and GPx4a at the periderm covering the yolk cell after 14-somite stage. Throughout early cleavages, GPx4 was located in blastomeres and was less abundant at the cleavage furrow. Later, from the 128-cell to 512-cell stages, GPx4 remained in the cytoplasm but gradually increased in the nuclei, beginning in marginal blastomeres and extending the nuclear localization to all blastomeres. During epiboly progression, GPx4b was found in blastoderm cells and was excluded from the yolk cell. After 24 h of development, GPx4b was present in the myotomes particularly in the slow muscle fibers, and was excluded from the myosepta. These results highlight the dynamics of the GPx4 localization pattern and suggest its potential participation in fundamental developmental processes.

1. Introduction

Reactive oxygen species (ROS) are natural oxygen byproducts of aerobic metabolism (Halliwell and Cross, 1994). Extensive evidence indicates that ROS act as second messengers in the control of major cellular behaviors, such as cellular proliferation, death, migration, differentiation and aging (Burdon and Rice-Evans, 1989; Harman, 1956; Sohal and Allen, 1990; Bouzyk et al., 1991; Rattan et al., 1997; Covarrubias et al., 2008); however when produced in excess, ROS have been implicated in different pathologies, including cancer, diabetes and cardiovascular and degenerative diseases (Halliwell and Whiteman, 2004). To metabolize and modulate ROS effects, organisms use a variety of non-enzymatic and enzymatic antioxidant defenses, including superoxide dismutases (Sod1, 2 and 3), catalases and glutathione peroxidases (GPx1, 2, 3 and 4). In mice, the targeted disruption of some of these enzymes, such as catalase (Ho et al., 2004), Sod1, 2 and 3 (Lebovitz et al., 1996; Elchuri et al., 2005; Carlsson et al., 1995) and GPx1, 2 and 3 (Esworthy et al., 2001; Ho et al., 1997; Olson et al., 2010), does not produce any visible developmental phenotypes. In contrast, glutathione peroxidase 4 (GPx4) knock-out mice present mid-gestation lethality between days 7.5 and 8.5 (Yant et al., 2003; Imai et al., 2003), highlighting the relevance of this particular antioxidant enzyme during development. Glutathione peroxidases (GPxs) 1 to 4 are a group of antioxidant proteins with selenium in their active sites that catalyze the reduction of hydroperoxides to water or their corresponding alcohols (Halliwell and Cross, 1994). GPx4 is the only glutathione peroxidase that functions as a monomeric protein, compared with the other GPxs that act as tetramer proteins (Ursini et al., 1985). GPx4 is capable of reducing small peroxides such as

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H_2O_2, and large ones, such as phospholipid and cholesterol hydroperoxides (Ursini et al., 1985; Thomas et al., 1990a, 1990b; Sattler et al., 1994). Interestingly, the GPx4 protein has been found in different sub-cellular locations, including the cytoplasm, the nucleus, the mitochondria and the endoplasmic reticulum, in different cultured cell lines (Arai et al., 1999). In mammals, the *Gpx4* gene presents at least three different alternative transcripts that code for proteins with different N-terminal sequences that determine their sub-cellular localization (Pushpa-Rekha et al., 1995; Ufer and Wang, 2011). Although the mode of action of GPx4 is well known, details of its expression during development are limited to the transcriptional level in mice (Borchert et al., 2006; Schneider et al., 2006) and in zebrafish (Thisse et al., 2003). Previously, we reported that the spatial restriction of GPx4 activity in mouse embryonic limbs protects the forming digits from cell death by limiting ROS accumulation to the interdigital tissues that regress during digit individualization (Schnabel et al., 2006). This previous evidence suggests that antioxidant enzymes participate in important developmental processes. Two different GPx4 genes, *gpx4a* and *gpx4b*, were previously identified in the zebrafish genome. Both genes were reported to show mutually exclusive expression patterns during late development from 1 to 5 days post fertilization embryos. *gpx4a* is expressed in the yolk cell and *gpx4b* in the embryo proper (Thisse et al., 2003). However the pattern of expression during the first 24 h of zebrafish development was not reported.

In this study, first we analyzed the gene expression of *gpx4a* and *gpx4b* by polymerase chain reaction (PCR) and found that *gpx4a* shows only zygotic expression that starts at shield stage. In contrast, *gpx4b* mRNA showed maternal inheritance and zygotic expression during the entire first 24 h of development. By *in situ* hybridization we confirmed the exclusive expression and localization of *gpx4a* transcripts in the periderm that cover the yolk cell, and the localization of *gpx4b* transcripts in the blastoderm cells. Consequently we consider that the GPx4 protein immunolocalization here reported mainly represents the GPx4b protein pattern in the embryo during the first 24 h of zebrafish embryo development, and GPx4a is observed in periderm covering the yolk cell after 14-somite stage. We found that the GPx4 protein presents specific patterns at particular developmental stages. For example, after fertilization during the early cleavages, GPx4 was localized in all blastomeres, but the protein signal decreases at the cleavage furrow. By the 128- to 512-cell stages, GPx4 localizes in the cytoplasm of all blastomeres but presents a distinct nuclear localization. This nuclear localization was observed in clusters of marginal blastomeres and eventually extended into the whole embryo. During epiboly, the GPx4b signal was found in the blastoderm and was excluded from the yolk cell. After 24 h of development, GPx4b was found at the myotomes with a decreased signal at the myosepta. In myotomes, GPx4b was particularly observed in slow muscle fibers. These patterns provide evidence that the GPx4 protein exhibits a dynamic localization during zebrafish development, suggesting that it participates in different events during vertebrate development.

2. Materials and methods

2.1. Fish maintenance and strains

An AB-TU-WIK hybrid line and wild-type zebrafish (*Danio rerio*) embryos were obtained from natural crosses and raised at 28 °C. Embryo stages were determined by morphological criteria according to Kimmel and collaborators (Kimmel et al., 1995). Cleavage and early blastula embryos up to the 512-cell stage were more precisely assigned to a particular stage by counting the nuclei and/or the segregated anaphase sister chromosomes as previously described (Mendieta-Serrano et al., 2013). Zebrafish were handled in compliance with local animal welfare regulations, and all protocols were approved by the Comité de ética (Instituto de Biotecnología, UNAM).

2.2. Gene cloning and in situ hybridization

40 to 60 stage-matched embryos at 16- to 32-cell stage, sphere-dome, shield, 70%-epiboly, bud and at 24 h post fertilization (hpf) were treated with 1 mL of RNAlater (Invitrogen) overnight. Total RNA was isolated by standard Trizol protocol (Invitrogen) following the manufacturer protocol. Genomic DNA was removed from the RNA samples by the TURBO DNA-free kit (Invitrogen). cDNA was generated with the M-MLV reverse transcriptase (Invitrogen) using a combination of Oligo (dT) (Invitrogen) and random primers (Invitrogen) in the cDNA synthesis cocktail. Oligonucleotides were designed from the reported reference transcript sequences from *gpx4a* and *gpx4b* (NM_001007282.1 and NM_001030070.2 respectively) to amplify the sequences by PCR reactions. The oligo sequences used are as follows. *gpx4a* primers 5′ AATTCGCTG TCTGCTGTTTCAG 3′ and 5′ TCCTCAACAAGGGGTTTCCA 3′. *gpx4b* primers 5′ TAGTATGTGGTTGTTTCAGAGAGC 3′ and 5′ GCCCAAAA-CACAGGACGGAG 3′. *18s* ribosomal RNA primers (McCurley and Callard, 2008) 5′ TCGCTAGTTGGCATCGTTTATG 3′ and 5′ CGGAGGT TCGAAGACGATCA 3′. The PCR products were cloned in the pCR II TOPO (Invitrogen), sequenced and used for *in situ* probe synthesis. The RNA *in situ* hybridization was performed by reported standard protocols (Thisse and Thisse, 2008).

2.3. Immunofluorescence

Whole-mount immunostaining in zebrafish embryos was used to determine the localization patterns of GPx4 as previously described (Mendieta-Serrano et al., 2013) with slight modifications. Freshly collected embryos at different stages of development were fixed overnight (o.n.) at 4 °C in 4% PFA in PBS, except for embryos at somite stages and 24 h of development that were fixed for 2 h at room temperature. In embryos that were extending their tails, the chorion was manually removed before fixation. Afterwards, the samples were washed 3 times in blocking buffer (PBS, BSA 0.1%, triton X100 1%), manually dechorionated and blocked for at least 5 h in blocking buffer at room temperature. The blocking step and all following steps were conducted with soft constant shaking. The primary antibody, rabbit IgG anti-GPx4 (H-90, sc-50497, Santa Cruz Biotechnology), was diluted 1:100 in blocking buffer (final concentration 2 μg/ml), and the embryos were incubated in this solution at 4 °C o.n. Subsequently, the embryos were washed 3 times in blocking buffer and were incubated in the secondary antibody goat anti-rabbit Alexa Fluor 647 (A-21244, Molecular Probes) diluted 1:100 in blocking buffer (final concentration 20 μg/ml) for 8 h at 4 °C. The embryos were washed 3 times in blocking buffer, and different fluorophores were used for counterstaining different structures. The selected dyes allowed for the simultaneous visualization and spectral resolution of different signals by confocal microscopy. To visualize actin filaments, embryos were stained with phalloidin Alexa Fluor 488 (A12379, Molecular Probes) diluted 1:100 in blocking buffer and incubated at 4 °C o.n. The embryos were washed 3 times in blocking buffer and treated with 0.4 mg/ml RNase (Roche) in blocking buffer for 1 h at 37 °C, washed 3 times in blocking buffer and stained with Hoechst 33258 (H1398, Molecular Probes) diluted 1:4000 in blocking buffer (final concentration 2.5 μg/mL) to visualize the DNA and nuclei. The embryos were then washed 3 times and mounted in low melting point agarose in PBS for confocal laser scanning microscopy.